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13. ABSTRACT *(Maximum 200 Words)* Disruptions of transforming growth factor-beta (TGF-ß) signaling contribute to the development and progression of human breast cancer. TGF-ß signals through its interaction with two TGF-ß type II receptors (TßRII) and subsequent recruitment of two type I receptors. In turn, parallel downstream signaling pathways activate SMAD proteins contributing to growth inhibition, as well as RhoA, p38MAPK, and PI3-kinase/AKT pathways involved in epithelial to mesenchymal transdifferentiation (EMT). We hypothesized that there may be a different TßRII activation threshold level required for the individual parallel downstream-signaling pathways. We disrupted TGF-ß signaling in mammary epithelial cells in culture and in mice by the expression of a dominant-negative TßRII (DNIIR). We found that similar DNIIR expression levels resulted in the inhibition of both TGF-ß-mediated growth inhibition and EMT. However, the threshold for TGF-ß-mediated SMAD and PI3-kinase/AKT activation was inhibited at a lower DNIIR expression level than that required for inhibiting the p38MAPK pathway. The transgenic expression of DNIIR in mice resulted in the development of spontaneous tumors. Interestingly DNIIR expression was undetecible specifically in the invasive tumors. Together the results support our initial hypothesis of differential TßRII activation thresholds and further suggest a role for TGF-ß in EMT potentially to facilitate metastasis.

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INTRODUCTION

TGF-ß was initially described as a secreted factor that induced an anchorage-independent transformed phenotype in fibroblasts *in vitro* (1). TGF-ß also induces phenotypic changes in epithelial cells and activates transcription of integrin genes as well as their corresponding extracellular matrix (ECM) ligands. The spectrum of TGF-ß responses are initiated by its interaction with cell surface receptors that form a heterotetrameric complex of two type I and two type II serine/threonine kinase receptors (2, 3). The type II receptor (TßRII) binds to the TGF-ß ligand to recruit and phosphorylate the type I receptor (3). The activated ligand-receptor complex in turn conveys the signal to the nucleus via the phosphorylation and activation of the SMAD signaling pathway (4). TGF-ß also stimulates parallel downstream signaling pathways involving RhoA (5), stress kinases (*i.e.* JNK, p38MAPK) (6-8), phosphatase2A (9), and PI3-kinase/AKT (10). Despite the cross-talk among the various signaling pathways, there is compelling data to suggest a separation of function among the individual signaling molecules [for review, see (11)]. Current evidence suggests that the SMAD pathway is involved in growth inhibition while the Rho proteins, and PI3-kinase pathways are likely operative in phenotypic changes (5, 12, 13).

Disrupted TGF-ß signaling is detected in neoplastic tissues, and specifically mutational inactivation in the TßRII through microsatellite instability (MSI) are frequent early events in human cancers of the prostate, colon, and breast. In order to isolate the role of TGF-ß we disrupted TGF-ß signaling by inducibly expressing a cytoplasmically truncated TßRII [DNIIR, (14)] in non-transformed mouse mammary epithelial (NMuMG) cells and in the mammary glands of transgenic mice. We hypothesized that there may be differential TGF-ß signaling as a result of DNIIR expression.

Epithelial cell adhesion and communication with the ECM and neighboring cells play fundamental roles in epithelial transdifferentiation into a mesenchymal phenotype (EMT). EMT has been defined by the loss of cell polarity, break down of cell-cell junctions, and the acquisition of an elongated cell shape. While tight junctions and adherence junctions mediate epithelial cell-cell interactions, its interaction with the substratum is through the heterodimeric integrin receptors (15). However, little is known about the underlying cross talk of TGF-ß and integrin signaling. We hypothesized that there may be cooperation between TGF-ß and integrins in both signal transduction and the modulation of EMT progression.

BODY

Regulation of DNIIR expression in NMuMG cells: We previously reported on the generation of an ecdysone-inducible NMuMG clone where we were able to regulate the expression of DNIIR. These cells were further characterized for their ability to undergo EMT and induction of specific signal transduction pathways. These results are detailed in our recent publicaton (16). In summary, the expression of DNIIR inhibited EMT and growth inhibition at similar expression levels, contrary to our initial hypothesis. However, there was differential TGF-ß-mediated downstream signaling as a result of DNIIR expression. We found that TGF-ß-mediated p38MAPK signaling was not blocked under DNIIR expression, while Smad, and AKT signals were attenuated. We further found that p38MAPK signaling by TGF-ß could be regulated by integrin ß1 activity. A large body of literature shows that the expression of integrins is regulated by TGF-ß, in particular the up-regulation of ß1 integrins (17-19). In addition, integrin-mediated adhesion potentiates many of the same signaling pathways regulated by TGF- ß such as the PI3-kinase-AKT, stress kinases, and ERK1/2 stimulated pathways, (20). When integrin ß1 was blocked TGF-ß could no longer activate p38MAPK. Finally we found that integrinß1 and p38MAPK signaling are necessary but not sufficient components of TGF-ß-mediated EMT in NMUMG cells.

MMTV-DNIIR expression in mice: In an effort to determine the consequence of TGF-ß signaling in mammary glands *in vivo*, we over expressed DNIIR in mice under the control of the MMTV promoter. We have been trying to characterize the mammary tumors that develop in mice expressing DNIIR in the mammary epithelia through the stimulation of the MMTV promoter. Our preliminary studies show that tumors form in the mouse close to one year of age. The long latency for tumor formation makes it difficult to have significant numbers of mice for us to examine at this point. However, we have observed most tumors not to be invasive and be characterized as ductal carcinoma *in situ*. Interestingly, however the tumors that have shown invasion or invasive potential have lost the expression of the DNIIR transgene at the leading edge according to *in situ* hybridization. The pathological assessments were made by H&E staining.

Some of the DNIIR expressing mammary tumors were cultured to better examine signal transduction pathways as performed for DNIIR expressing NMuMG cells above. These studies revealed that the transgene expression diminished as the cells were passaged analogous to what was seen in the aggressive tumors in the mice. The primary mammary cultures were refractile to TGF-ß-mediated growth inhibition as well as resistant to SMAD and AKT activation, but able to activate p38MAPK. After 18 passages the cells were TGF-ß responsive to growth, SMAD, AKT and p38MAPK signaling. These studies are on going and require more tumors to support our current findings.

TGF-ß-mediated RhoA signaling: Since we initially identified RhoA signaling to be an important component of TGF-ß-mediated EMT progression, cDNA constructs were engineered to express dominant negative and wild type RhoA in the ecdysone regulatible system. We found that the expression of dominant negative RhoA in NMuMG cells results in the inhibition of TGF-ß-mediated RhoA activation and inhibited EMT

progression. We do not plan on pursuing the regulation of Rac1, RhoB, or JNK1 by the ecdysone system, since these signaling molecules did not seem to play a critical role in TGF-ß-mediated EMT progression. Instead we feel the role of RhoA activation in TGF-ß signaling needs to be better understood. Since the dominant negative RhoA construct has the capacity to affect the activity of other GTPases we have designed a rhotekin binding domain cDNA construct for mammalian cell expression. This has been shown to be very specific for blocking RhoA signaling (21). Thus we plan on using this construct instead of the RhoA dominant negative construct for future studies.

KEY RESEARCH ACCOMPLISHMENTS:

- Characterized the integrin expression in NMuMG cells to be primarily α2ß1, α5ß1, and αvß1. These specific integrins are receptors for collagen, fibronectin, and vitronectin, respectively.
- Identified integrin ß1 signaling to be an essential component in TGF-ß signaling and EMT progression.
- Identified the role of p38MAPK in TGF-ß signaling to be involved in EMT progression.
- Isolated mammary tumors from MMTV-DNIIR transgenic mice and examined paraffin sections by H&E staining and DNIIR expression by in situ hybridization.
- Developed and partially characterized primary mouse mammary cell lines expressing DNIIR.

REPORTABLE OUTCOMES:

Research

Manuscript

- **Bhowmick, N.A.**, Zent R, Ghiassi M, McDonnell M, Moses H.L. (2001) Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity. *J Biol Chem.* 276:46707-13.

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- Ortho-McNeil Young Investigator Award
- Aventis Pharmaceuticals, Leadership Development in Cancer Research Program Award

Products

CDNA construct, Cell lines and animal models developed

- Stable NMuMG cells expressing dominant-negative p160ROCK were generated.
- Mammalian expression constructs for the rhotekin RhoA binding domain were made.
- Developed 6 primary mouse mammary cell lines expressing DNIIR.

CONCLUSIONS:

In conclusion we have data indicating that TGF-ß can stimulate p38MAPK activation at a lower functional TßRII threshold than the activation of Smad2 and AKT. Furthermore, TGF-ß dependent p38MAPK activation is dependent on integrin ß1-ECM interactions and TGF-ß-induced epithelial cell EMT is both p38MAPK activation and integrin-ß1-mediated. Thus far we have identified RhoA, PI3-kinase/AKT, and p38MAPK activity as important components of TGF-ß signaling contributing to the complex process of EMT. Our preliminary data examining the expression of DNIIR in the various tumors further suggest the importance of activation of the full spectrum of TGF-ß signaling in the process of tumor invasion. Taken together these data indicate that functional TßRII dosage as well as cooperative signaling between the TGF-ß receptor and integrins may influence the phenotypic fate of epithelial cells *in vivo*.

REFERENCES:

1. Moses, H. L., Branum, E. L., Proper, J. A. & Robinson, R. A. (1981) *Cancer Res* **41,** 2842-8.
2. Ebner, R., Chen, R. H., Shum, L., Lawler, S., Zioncheck, T. F., Lee, A., Lopez, A. R. & Derynck, R. (1993) *Science* **260,** 1344-8.
3. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F. & Massague, J. (1994) *Nature* **370,** 341-7.
4. Zhang, Y., Feng, X., We, R. & Derynck, R. (1996) *Nature* **383,** 168-72.
5. Bhowmick, N. A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C. A., Engel, M. E., Arteaga, C. L. & Moses, H. L. (2001) *Mol Biol Cell* **12,** 27-36.
6. Atfi, A., Djelloul, S., Chastre, E., Davis, R. & Gespach, C. (1997) *J Biol Chem* **272,** 1429-32.
7. Engel, M. E., McDonnell, M. A., Law, B. K. & Moses, H. L. (1999) *J Biol Chem* **274,** 37413-20.
8. Wang, W., Zhou, G., Hu, M. C., Yao, Z. & Tan, T. H. (1997) *J Biol Chem* **272,** 22771-5.
9. Griswold-Prenner, I., Kamibayashi, C., Maruoka, E. M., Mumby, M. C. & Derynck, R. (1998) *Mol Cell Biol* **18,** 6595-604.
10. Krymskaya, V. P., Hoffman, R., Eszterhas, A., Ciocca, V. & Panettieri, R. A., Jr. (1997) *Am J Physiol* **273,** L1220-7.
11. Massague, J. (2000) *Nat Rev Mol Cell Biol* **1,** 169-78.
12. Ashcroft, G. S., Yang, X., Glick, A. B., Weinstein, M., Letterio, J. L., Mizel, D. E., Anzano, M., Greenwell-Wild, T., Wahl, S. M., Deng, C. & Roberts, A. B. (1999) *Nat Cell Biol* **1,** 260-6.
13. Bakin, A. V., Tomlinson, A. K., Bhowmick, N. A., Moses, H. L. & Arteaga, C. L. (2000) *J Biol Chem* **275,** 36803-10.
14. Chen, R. H., Ebner, R. & Derynck, R. (1993) *Science* **260,** 1335-8.
15. Boudreau, N. J. & Jones, P. L. (1999) *Biochem J* **339,** 481-8.
16. Bhowmick, N. A., Zent, R., Ghiassi, M., McDonnell, M. & Moses, H. L. (2001) *J Biol Chem.*
17. Kumar, N. M., Sigurdson, S. L., Sheppard, D. & Lwebuga-Mukasa, J. S. (1995) *Exp Cell Res* **221,** 385-94.
18. Roberts, C. J., Birkenmeier, T. M., McQuillan, J. J., Akiyama, S. K., Yamada, S. S., Chen, W. T., Yamada, K. M. & McDonald, J. A. (1988) *J Biol Chem* **263,** 4586-92.
19. Ignotz, R. A. & Massague, J. (1987) *Cell* **51,** 189-97.
20. Mainiero, F., Soriani, A., Strippoli, R., Jacobelli, J., Gismondi, A., Piccoli, M., Frati, L. & Santoni, A. (2000) *Immunity* **12,** 7-16.
21. Welsh, C. F., Roovers, K., Villanueva, J., Liu, Y., Schwartz, M. A. & Assoian, R. K. (2001) *Nat Cell Biol* **3,** 950-7.

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Integrin β_1 Signaling Is Necessary for Transforming Growth Factor-β Activation of p38MAPK and Epithelial Plasticity*

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Transforming growth factor-β (TGF-β) can induce epithelial to mesenchymal transdifferentiation (EMT) in mammary epithelial cells. TGF-β-meditated EMT involves the stimulation of a number of signaling pathways by the sequential binding of the type II and type I serine/threonine kinase receptors, respectively. Integrins comprise a family of heterodimeric extracellular matrix receptors that mediate cell adhesion and intracellular signaling, hence making them crucial for EMT progression. In light of substantial evidence indicating TGF-β regulation of various β_1 integrins and their extracellular matrix ligands, we examined the cross-talk between the TGF-β and integrin signal transduction pathways. Using an inducible system for the expression of a cytoplasmically truncated dominant negative TGF-β type II receptor, we blocked TGF-β-mediated growth inhibition, transcriptional activation, and EMT progression. Dominant negative TGF-β type II receptor expression inhibited TGF-β signaling to the SMAD and AKT pathways, but did not block TGF-β-mediated p38MAPK activation. Interestingly, blocking integrin β_1 function inhibited TGF-β-mediated p38MAPK activation and EMT progression. Limiting p38MAPK activity through the expression of a dominant negative-p38MAPK also blocked TGF-β-mediated EMT. In summary, TGF-β-mediated p38MAPK activation is dependent on functional integrin β_1, and p38MAPK activity is required but is not sufficient to induce EMT.

TGF-β[1] has emerged as a multifunctional cytokine involved in autocrine and paracrine mediation of development, proliferation, wound healing, and pathologic processes (1). TGF-β was initially described as a secreted factor that induced an anchorage-independent transformed phenotype in fibroblasts *in vitro* (2). TGF-β also induces phenotypic changes in epithelial cells and activates transcription of integrin genes as well as their extracellular matrix (ECM) ligands. The spectrum of TGF-β responses are initiated by its interaction with cell surface receptors that form a heterotetrameric complex of two type I and two type II serine/threonine kinase receptors (3, 4). The type II receptor binds to the TGF-β ligand to recruit and phosphorylate the type I receptor (4). The activated ligand-receptor complex in turn conveys the signal to the nucleus via the phosphorylation and activation of the SMAD signaling pathway (5). TGF-β also stimulates parallel downstream signaling pathways involving RhoA (6), stress kinases (*i.e.* JNK, p38MAPK) (7–9), phosphatase2A (10), and PI3-kinase/AKT (11). Despite the cross-talk among the various signaling pathways, there is compelling data to suggest a separation of function among the individual signaling molecules (for review, see Ref. 12). Current evidence suggests that the SMAD pathway is involved in growth inhibition, while the Rho proteins, and PI3-kinase pathways are likely operative in phenotypic changes (6, 13, 14).

Epithelial cell adhesion and communication with the ECM and neighboring cells play fundamental roles in epithelial transdifferentiation into a mesenchymal phenotype (EMT). EMT has been defined by the loss of cell polarity, break down of cell-cell junctions, and the acquisition of an elongated cell shape. While tight junctions and adherence junctions mediate epithelial cell-cell interactions, its interaction with the substratum is through the heterodimeric integrin receptors (15). A large body of literature shows that the expression of integrins is regulated by TGF-β, in particular the up-regulation of β_1 integrins (16–18). In addition, integrin-mediated adhesion potentiates many of the same signaling pathways regulated by TGF-β such as the PI3-kinase-AKT, stress kinases, and ERK1/2-stimulated pathways, (19). However, little is known about the underlying cross talk of TGF-β and integrin signaling.

Given the convergence of TGF-β and integrin signaling on common downstream factors as well as their central effects on cytoskeletal organization, we hypothesized that there may be cooperation between TGF-β and integrins in both signal transduction and the modulation of EMT progression. To isolate the role of TGF-β we disrupted TGF-β signaling by inducibly expressing a cytoplasmically truncated TβRII (DNIIR, (20)) in non-transformed mouse mammary epithelial cells, NMuMG. Elevated expression of DNIIR caused the loss of TGF-β responsiveness to growth, SMAD-mediated transcriptional regulation, and EMT progression without the apparent loss of p38MAPK activation. However, blocking integrin β_1 adhesion inhibited TGF-β-mediated p38MAPK stimulation and EMT progression. The findings suggest integrin β_1 adhesion and p38MAPK signaling are necessary but not sufficient for the complex process of TGF-β-induced EMT.

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[1] The abbreviations used are: TGF, transforming growth factor; ECM, extracellular matrix; JNK, c-Jun NH_2-terminal kinase; PI, phosphatidylinositol; EMT, epithelial to mesenchymal transdifferentiation; DNIIR, cytoplasmically truncated dominant negative TGF-β type II receptor; MAPK, mitogen-activated protein kinase; DNp38MAPK, dominant negative p38MAPK.

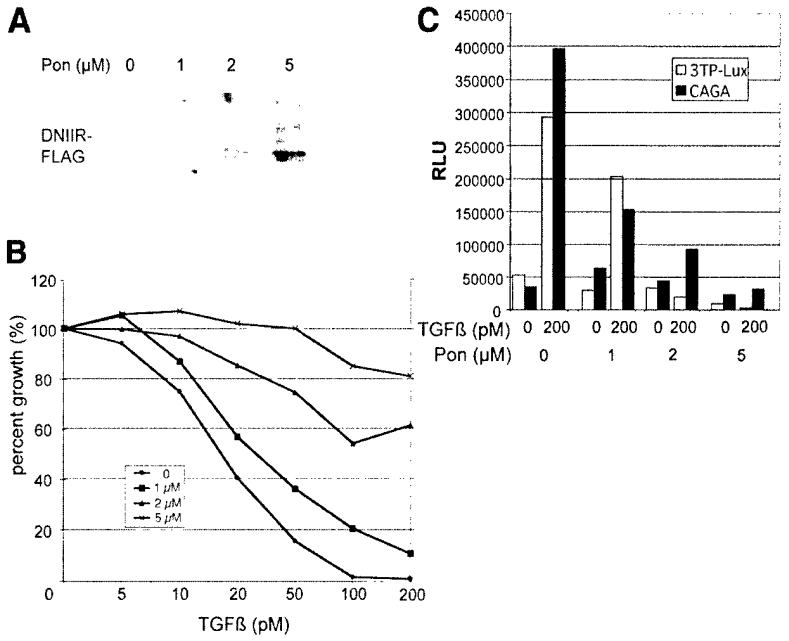

FIG. 1. **Inducible expression of DNIIR in NMuMG cells indicated concentrations of ponasterone (*Pon*) were added to cells 24 h prior to assaying for protein expression, TGF-β-mediated growth inhibition, or TGF-β-induced transcriptional activation.** *A*, ponasterone-induced cells were harvested for immunoblotting with anti-FLAG antibody. This is representative of three blots for the FLAG-tagged DNIIR. *B*, increasing concentrations of TGF-β were incubated (24 h) with ponasterone-induced cells and assayed for [^{3}H]thymidine incorporation (in triplicate, $n = 2$). The graph is represented as a percent of growth, having the absence of TGF-β expressed as 100%. *C*, the activation of the 3TP-Lux and CAGA luciferase-conjugated reporters were assayed after TGF-β treatment for 24 h (in triplicate, $n = 2$).

MATERIALS AND METHODS

Cell Culture—The NMuMG and T47D cells were purchased from the American Type Culture Collection (Manassas, VA) and propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 μg/ml insulin. The ecdysone-inducible mammalian expression system was used to regulate the expression of the *DNIIR* gene (a kind gift from R. Derynck, (20)) in NMuMG cells as described by the manufacturer (Invitrogen Co., Carlsbad, CA). Briefly, clones of stable lines expressing the pVgRXR plasmid (containing the ecdysone receptor) were generated and selected for maximal expression. Next, the *DNIIR* cDNA in the inducible expression plasmid (pIND) was introduced into the selected pVgRXR-expressing clone. Finally, NMuMG clones expressing both plasmids were identified by sensitivity to TGF-β (by growth inhibition and transcriptional reporter assays). Ponasterone A (Invitrogen Co., 0–5 μM) was added to the cells 24 h prior to experiments to achieve the desired DNIIR expression level. Ponasterone (5 μM) was added to wild type NMuMG cells (not transfected with the pIND vector) to examine its effects on TGF-β-mediated epithelial differentiation and p38MAPK activation. No ponasterone-associated results were observed (data not shown).

Virus Generation—The *DNIIR* cDNA was subcloned into pBabe retroviral vector. Then both *DNIIR* and *TβRII* (kindly provided by W. Grady, Vanderbilt University) retrovirus constructs were transfected into amphetrophic retrovirus producing Phoenix cells (a gift from Gary Nolan, Stanford University). Conditioned media was allowed to incubate with target cells for 24 h, after which point the media was replaced for subsequent experiments. The DNp38MAPK adenovirus (21) was amplified in human embryonic kidney cells, HEK293, cesium chloride-purified and titrated by serial dilution (Takara Shuzo Co. Ltd.). Experiments were performed 48 h after retrovirus or adenovirus infection.

Thymidine Incorporation and Luciferase Reporter Assay—Cell growth was assayed by [^{3}H]thymidine (PerkinElmer Life Sciences) incorporation of cells treated for 24 h with TGF-β and loaded with [^{3}H]thymidine 2 h prior to harvesting (22). Cells were washed and measured by scintillation counting. Transcriptional activation was tested in cells transfected with the luciferase (firefly) reporter construct cDNAs indicated in conjunction with a cytomegalovirus-driven renal luciferase plasmid (Promega Inc.). The 3TP-Lux reporter from J. Massagué (Memorial Sloan-Kettering Cancer Center, NY) and CAGA reporter from J.-M. Gauthier (Laboratoire Glaxo Wellcome, Les Ulis Cedex, FranceTranscriptional) were used to asses TGF-β-signaling to the nucleus in the presence and absence of DNIIR expression. The dual-luciferase assays were performed on lysed cells as indicated by the manufacturer, Promega Inc. The firefly luciferase activity determined by luminometer measurements were normalized to renela luciferase activity, and data is reported in relative luminescent units.

Immunoprecipitation and Immunoblot Analysis—Serum-starved cells were preincubated with or without integrin β_1 antibody (Ha2/5, BD PharMingen, San Diego, CA) for 4 h and subsequently treated with TGF-β for the times indicated. The cells were then lysed by sonication in 25 mM HEPES buffer, pH 7.5, (containing 300 mM NaCl, 20 mM β-glycerol phosphate, 2 mM sodium pyrophosphate, 1 mM EDTA, 0.2 mM EGTA, 0.1% SDS, 1% Triton X-100, 10% glycerol, 1 mM sodium vanadate, 10 nM microcystin, and 1 mM phenylmethylsulfonyl fluoride), and equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis for subsequent immunodetection by blotting on to polyvinylidene difluoride membranes. The immunoblots were visualized by either enhanced chemiluminescence (ECL) detection (Amersham Pharmacia Biotech) or alkaline phosphatase colorimetric development.

Kinase Assays—Cell extracts (400 μg of protein) in HEPES buffer were subjected to immunoprecipitation by an antibody to p38MAPK (C-20, Santa Cruz Biotechnology) for 2 h at 4 °C, followed by incubation with protein G-Sepharose (Sigma) for 1 h. The beads were washed and resuspended in 3 μM [γ-^{32}P]ATP, 100 μM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM β-glycerol phosphate, and ATF2 as substrate. The assay was allowed to incubate at 30 °C for 20 min prior to denaturation in Laemmli buffer and separation on a 5–20% gradient polyacrylamide gel. The gel was then fixed and visualized by phosphorimaging.

Immunofluorescence—Cells grown on glass coverslips with or without collagen I coating (10 μg/ml, Collaborative Labs) were fixed with 100% cold methanol for immunolocalization of ZO-1 (Chemicon) and E-cadherin (Santa Cruz Biotechnology) or by 4% paraformaldehyde for Smad1/2/3 (Santa Cruz Biotechnology). Subsequently PBS containing 0.1% Triton X-100 and 1% horse serum was used for permeabilization and blocking. The primary antibody (1:1000) was incubated for 1 h, and Cy3 (Sigma) secondary antibody was used for fluorescence visualization. F-actin and nuclear staining was done by fixing the cells in 4% paraformaldehyde followed by incubation with Texas-Red-conjugated phalloidin (Molecular Probes, Eugene, OR) or Hoechst 33253, respectively.

RESULTS

DNIIR Expression—When the cytoplasmic region of TβRII is truncated and lacks the serine/threonine kinase domain it is able to bind TGF-β and recruit the type I receptor, but is unable to signal via the SMAD signaling pathway, and is thus termed a dominant negative TβRII, DNIIR (20). We sought to generate stable clones in non-transformed mouse mammary ductal epithelial cells, NMuMG, that have an inducible *DNIIR* gene to identify down stream signaling pathways that may be differentially regulated by TGF-β. Fig. 1*A* shows an increase in FLAG-tagged DNIIR protein expression that corresponds to increasing concentrations of ponasterone (an ecdysone analogue). To verify that the expression of DNIIR resulted in a functional loss of growth inhibitory and SMAD-mediated transcriptional activation by TGF-β, as reported earlier (20), thymidine incorporation assays and transcriptional reporter as-

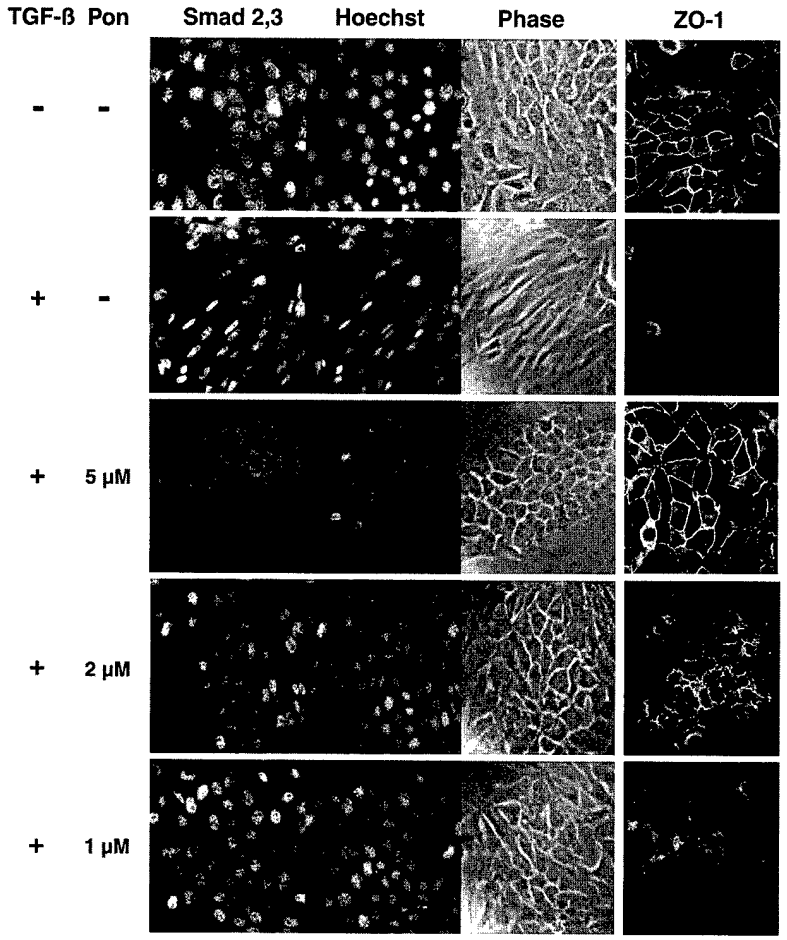

FIG. 2. **Epithelial differentiation blocked by DNIIR expression.** NMuMG cells grown on glass coverslips were preincubated with ponasterone (0–5 μM) followed by TGF-β (0 or 200 μM) treatment. Cells were then fixed and stained for the nucleus (with Hoechst), Smad 2,3, or *ZO-1*. Images were captured using phase contrast and fluorescence microscopy.

says were performed, respectively. As the DNIIR expression was induced with 0–5 μg/ml ponasterone, TGF-β-mediated growth inhibition was essentially abolished by 2 and 5 μM ponasterone (Fig. 1*B*). Next, the role of DNIIR expression in TGF-β-mediated transcriptional activation was examined by 3TP-lux (the heterologous PAI-1 promoter with 3× 12-*O*-tetradecanoylphorbol-13-acetate-responsive elements (23)) and CAGA (the SMAD DNA binding domain (24)) luciferase reporter assays. The addition of TGF-β had a reduced induction on both the reporters when ponasterone was present (Fig. 1*C*). At 1 μg/ml ponasterone TGF-β transcriptional activation of 3TP-Lux was reduced 1.5-fold, and the CAGA activity by 2.7-fold. Treatment with 2 and 5 μM ponasterone resulted in TGF-β-mediated transcriptional activity that was reduced to basal levels. These results suggest that responses to TGF-β depending either solely on SMAD signaling or more complex signaling activating 3TP-Lux is blocked by the expression of DNIIR. Thus the expression of DNIIR in stable inducible clones cause cells to become insensitive to TGF-β-regulation of growth and SMAD-mediated transcriptional activation.

To further understand the repercussions of DNIIR expression in NMuMG cells we studied its role in TGF-β-mediated EMT. The expression of an apical tight junction-associated protein, ZO-1, was used as a marker to indicate cell polarity and cell-cell junction stability for the epithelial phenotype of NMuMG cells. We have previously shown that TGF-β-mediated fibroblastic conversion causes a distinct loss of ZO-1 expression at tight junctions (14). NMuMG cells in the presence and absence of ponasterone and TGF-β (200 pM for 16 h) were examined for immunolocalization of ZO-1 and Smad2/3, along with corresponding phase contrast images for phenotypic changes. Fig. 2 illustrates TGF-β-induced Smad2/3 nuclear localization accompanied by the loss of ZO-1 expression and the acquisition of a mesenchymal phenotype. Increasing expression levels of DNIIR reduced TGF-β-mediated NMuMG fibroblastic conversion and Smad2/3 nuclear localization. We were able to follow the sequential loss of junctional ZO-1 staining and subsequent acquisition of the elongated cellular phenotype as ponasterone concentrations were reduced in the presence of TGF-β. These results highlight the importance of the functionality of TβRII in TGF-β-mediated SMAD nuclear localization and the complexity of EMT progression.

Differential Signaling Activation in the Presence of DNIIR Expression—As EMT is a well described phenotypic consequence of TGF-β signaling, we investigated the activation states of various signaling proteins potentially involved in the inhibition of EMT by the expression of DNIIR in NMuMG cells. Fig. 3 shows that Smad2 phosphorylation occurs within 30 min of incubation with 200 pM TGF-β and maintains its phosphorylated status at 3 h. The expression of DNIIR, using 5 μM ponasterone, diminished TGF-β-mediated Smad2 phosphorylation. Total Smad2 levels did not change as a consequence of DNIIR expression over the time course. Next, we examined other signaling pathways activated by TGF-β. In the absence of DNIIR expression, cells showed modest TGF-β-induced p38MAPK and AKT (an indicator of PI3-kinase activity) phosphorylation with negligible ERK1/2 activation. TGF-β-mediated AKT phosphorylation was blocked by DNIIR expression. However, DNIIR expression did not block TGF-β-stimulated p38MAPK phosphorylation. DNIIR expression did not seem to alter ERK1/2 phosphorylation levels with respect to total ERK1/2 levels in the NMuMG cells. Cells were treated with EGF as a positive control for the activation of ERK1/2 and AKT. The mitogen-activated protein kinase inhibitor, PD98059

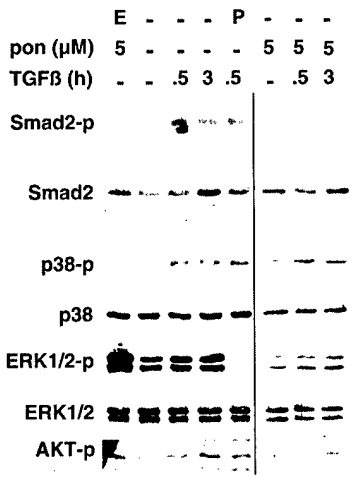

FIG. 3. **TGF-β-mediated signaling in the presence and absence of DNIIR expression.** *A*, NMuMG cells cultured in the absence (−) or presence of 5 μM ponasterone (*Pon*) and, 200 pM TGF-β were immunoblotted for phosphorylated forms and total Smad2, ERK1/2, p38MAPK, and phospho-AKT. Cells were incubated with EGF (*E*, 20 ng/ml for 30 min) as a positive control for ERK1/2 and AKT phosphorylation. PD98059 (*P*, 10 μM for 3 h) was used as a negative control for ERK1/2 phosphorylation.

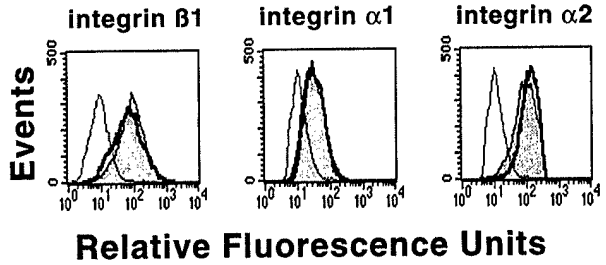

FIG. 4. **Effect of induced DNIIR expression on integrin expression.** NMuMG cells treated with ponasterone (5 μM) to express DNIIR (*black line*) or untreated (*filled*) were incubated in serum-free media for 24 h at which time cell surface integrin expression was measured by a fluorescence-activated cell sorter calibur, Becton Dickerson. An isotype-matched IgG was used as a control (*gray line*).

(Cell Signaling Technology), was used as a negative control. These results indicate that the receptor-ligand complex consisting of the type I TGF-β receptor and DNIIR results in the loss of downstream regulation of the AKT and SMAD signaling pathways but were ineffective in preventing TGF-β-stimulated phosphorylation of p38MAPK.

DNIIR Effects on Integrin Expression—A large body of evidence indicates that TGF-β signaling is involved in integrin expression. Thus it was important to determine whether the down-regulation of TGF-β autocrine signaling through the induced expression of DNIIR affects integrin expression. As NMuMG cells express the collagen receptor $\alpha_2\beta_1$ that is known to be important in integrin-mediated activation of p38MAPK (25), we assessed whether overexpression of DNIIR affected expression of this integrin. To do this, NMuMG cells were incubated for 24 h in serum-free medium in the presence or absence of DNIIR expression (by 5 μM ponasterone) after which the cells were subjected to flow cytometry. As seen in Fig. 4 no changes in the cell surface expression of this integrin was observed. Furthermore, there was also no alteration in expression of the other major collagen-integrin, $\alpha_1\beta_1$. These results suggest that overexpression of DNIIR for 24 h does not alter the expression levels of the major collagen binding integrins in NMuMG cells under serum-free conditions.

p38MAPK Activation by TGF-β Is Dependent on Integrin Ligation—Integrin β_1 ligation is known to induce p38MAPK phosphorylation and activation (19, 25). The inducible DNIIR expression system was ideal for examining if cooperative interactions exists between TGF-β and integrin signaling at the level of p38MAPK activity. Since NMuMG cells express the collagen I binding integrins, and the ligation of integrin $\alpha_2\beta_1$ by collagen I is known to activate p38MAPK, these cells were grown on collagen I to examine the specific role of β_1 integrins in TGF-β signaling. Under these growth conditions TGF-β induced Smad2 and p38MAPK phosphorylation as before (Fig. 5*A*). However, incubation of the cells with a neutralizing integrin β_1 antibody caused a decrease in p38MAPK phosphorylation but had no effect on TGF-β induction of Smad2 phosphorylation. Further, p38MAPK phosphorylation was observed when DNIIR-induced NMuMG cells were treated with TGF-β on either the tissue culture plates or collagen I; however, this was diminished by the co-incubation with an integrin β_1-neutralizing antibody (Fig. 5*B*). The neutralizing antibody did not alter the ERK1/2 or AKT responses to TGF-β (data not shown).

To determine whether the p38MAPK phosphorylation was integrin β_1-dependent and not simply adhesion-dependent we assessed the effect of TGF-β on NMuMG cells cultured on poly-L-lysine-coated plates. This allowed cells to attach based on electrostatic interactions, whereas cells plated onto collagen-coated dishes spread due to integrin engagement (26). Similar to the results seen with the integrin β_1-neutralizing antibody, TGF-β-mediated Smad2 phosphorylation was not effected by poly-L-lysine-mediated cell adhesion; however, there was no induction of TGF-β-mediated p38MAPK phosphorylation (Fig. 5*C*). These results suggest that integrin-mediated cell adhesion is required for TGF-β to stimulate p38MAPK phosphorylation, and TGF-β-induced Smad2 phosphorylation is independent of integrin-mediated adhesion.

The regulation of p38MAPK phosphorylation was then directly determined by examining kinase activity in the context of TβRII and integrin β_1 disruption. The *in vitro* kinase assay using immunoprecipitated p38MAPK from treated cells and ATF2 as substrate verified a TGF-β-dependent increase in p38MAPK activity in the presence and absence of ponasterone (Fig. 6). The preincubation of cells with the integrin β_1-neutralizing antibody prior to TGF-β stimulation diminished the levels of ATF2 phosphorylation. However, p38MAPK activation by ultraviolet light exposure was not effected by the integrin β_1 antibody, demonstrating that the requirement of integrin β_1 ligation was specific for TGF-β-induced p38MAPK activation. These results were consistent with p38MAPK phosphorylation previously observed (see Figs. 3 and 5) in confirming that DNIIR expression does not block TGF-β-mediated p38MAPK activation, and integrin β_1-mediated adhesion is necessary for TGF-β signaling to the stress kinase.

TβRII Expression Required for Differential Signaling Activation—Next we sought to determine whether TGF-β-mediated p38MAPK activation in our inducible system was mediated by the formation of a complex consisting of the type I TGF-β receptor and DNIIR or the type I TGF-β receptor and endogenous TβRII. To do this we expressed either DNIIR or TβRII by retroviral infection in a TβRII null mammary epithelial cell line, T47D. Not surprisingly, T47D cells were insensitive to TGF-β for SMAD and p38MAPK activity (Fig. 7). The transduction of DNIIR expression was not able to restore either SMAD or p38MAPK phosphorylation by TGF-β. However, TβRII transduction restored both TGF-β-induced Smad2 and p38MAPK phosphorylation. Further preincubation with integrin β_1 antibody blocked p38MAPK phosphorylation, but did not effect Smad2 activity, as observed with NMuMG cells. These results suggest that p38MAPK activation in the NMuMG cells is transduced by the endogenous type I and type II TGF-β receptor complex and that the level of DNIIR expres-

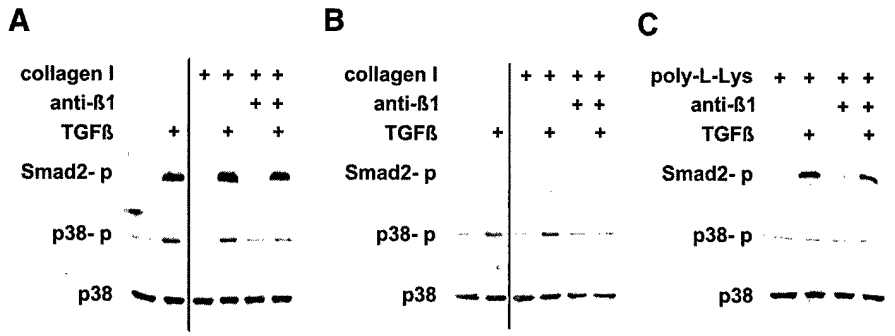

FIG. 5. **Adhesion-dependent TGF-β phosphorylation of p38MAPK.** NMu MG cells grown on plastic were replated on plastic, 10 μg/ml collagen I, or poly-L-lysine (0.1%) for 12 h prior to TGF-β (200 pM for 30 min) treatment. *A* and *B*, integrin β_1-neutralizing antibody was incubated for 4 h prior to TGF-β treatment. *B*, cells induced with 5 μM ponasterone were treated as indicated. Equivalent cell extracts were immunoblotted for phospho-Smad2, phospho-p38MAPK (Thr-180/Tyr-182), and p38MAPK.

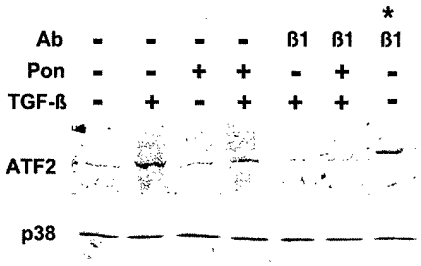

FIG. 6. **p38MAPK *in vitro* kinase assay.** Ponasterone induced or non-induced NMuMG cells, grown on collagen I, were preincubated with anti-integrin β_1 (4 h) prior to TGF-β (200 pM for 30 min) treatment. Cells were harvested, and equivalent protein amounts were immunoprecipitated with anti-p38MAPK antibody for subsequent evaluation of kinase activity. As a control for p38MAPK activation, cells were treated with 100 μJ of UV light 30 min prior to harvesting (indicated by *).

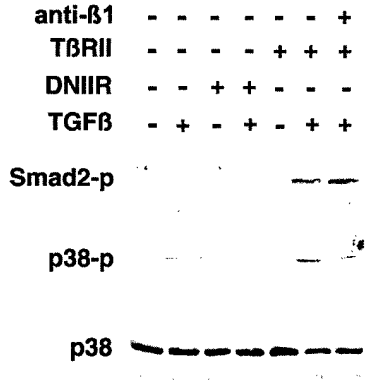

FIG. 7. **The expression of DNIIR and TβRII in T47D cells.** T47D cells transduced with TβRII, DNIIR, or empty retrovirus were incubated in the absence (−) or presence (+) of 200 pM TGF-β for 30 min prior to harvesting. Integrin β_1-neutralizing antibody was incubated for 4 h prior to TGF-β treatment. Equivalent cell extracts were immunoblotted for phospho-Smad2, phospho-p38MAPK, and p38MAPK.

sion achieved by the inducible system was insufficient to completely suppress endogenous TβRII activity. Thus the threshold of TβRII activity for p38MAPK activation may be lower than that for SMAD activation.

Integrin β_1 and p38MAPK Signaling Involved in TGF-β-mediated Phenotypic Change—Since integrin function appears to be involved in certain aspects of TGF-β signaling and is important for cell-ECM interactions that effect cell morphology (27), we examined whether integrin β_1 signaling affected TGF-β-induced EMT progression. NMuMG cells, grown on collagen I-coated coverslips, were incubated with integrin β_1-neutralizing antibodies or rabbit-IgG in the presence or absence of 200 pM TGF-β for 16 h under serum-free conditions. Cell phenotype was subsequently examined by phase contrast microscopy concurrent with actin, ZO-1, and E-cadherin subcellular localization by immunofluorescent detection (Fig. 8). When NMuMG cells were grown on collagen I, stress fiber formation was observed (Fig. 8, *inset*) accompanied by junctional localization of ZO-1 and E-cadherin. TGF-β treatment caused increased stress fiber formation and the acquisition of a fibroblastic morphology along with the delocalization of ZO-1 and E-cadherin. When cells were grown in the presence of anti-integrin β_1 antibody prominent cortical actin staining accompanied with reduced ZO-1 tight junctional localization was observed in untreated cells. The adhesion junctions, however, seemed to be intact with E-cadherin localization at the cell-cell contacts. Subsequent TGF-β treatment did not seem to alter the epithelial morphology of the cells in the presence of the integrin β_1 antibody. The incubation with rabbit IgG had little effect on the integrity of the cell junctions, and TGF-β caused a phenotypic differentiation to take place exhibiting the loss of ZO-1 and E-cadherin junctional localization (data not shown). Thus specifically disrupting integrin β_1 function inhibited TGF-β-induced EMT in NMuMG cells.

To determine whether blocking TGF-β-mediated p38MAPK activity may be a component in the inhibition of EMT by integrin β_1 neutralizing antibody, we transduced a dominant negative p38MAPK (DNp38MAPK-β, (21) Y. Wang, Univ. of California, San Diego) by adenovirus infection (20 multiplicity of infection). The expression of DNp38MAPK-β in NMuMG cells inhibited the TGF-β-induced fibroblastic transition. As a negative control, adenoviral expression of β-galactosidase did not show any noticeable differences in TGF-β-induced epithelial differentiation from the un-infected control (data not shown). The DNp38MAPK-β-mediated down-regulation of p38MAPK phosphorylation in NMuMG cells is shown in the *right panel* of Fig. 8. Thus limiting p38MAPK activity did not alter TGF-β-induced actin stress fiber formation, yet it resulted in the consistent localization of ZO-1 and E-cadherin at the cell junctions after 16 h of treatment with TGF-β. Together, these results suggest that impairing either integrin β_1 or p38MAPK signaling inhibit TGF-β-induced EMT progression in NMuMG cells. Furthermore, blocking EMT in NMuMG cells through the inhibition of integrin β_1 may in part be because it inhibits p38MAPK signaling.

DISCUSSION

The extracellular microenvironment plays a critical role in the ability of an epithelial cell to transition to a more plastic phenotype. This is exemplified by the influence that TGF-β and ECM components have one basic epithelial cell functions such as the morphology, polarity, and adhesion through TGF-β receptors and integrin function. Based on the common downstream signaling molecules and overlapping cellular consequences induced by TGF-β and integrins, we hypothesized that there may be cooperation between the two pathways in EMT. We utilized an inducible system expressing DNIIR in mammary epithelial cells and employed an integrin β_1 antibody to explore potential cross-talk between the two cell surface receptors. Effectively limiting TβRII activity by the induction of

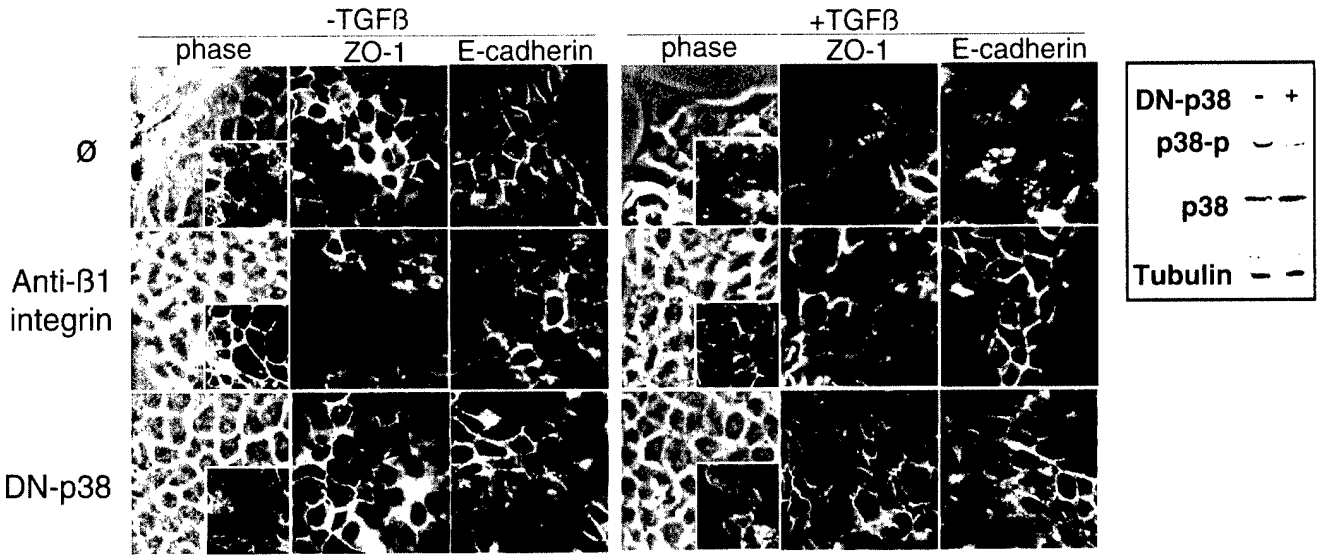

FIG. 8. **Adhesion- and p38MAPK-dependent TGF-β-mediated EMT.** NMuMG cells, cultured on collagen-coated coverslips, were incubated in serum-free media (ø) containing anti-integrin β_1 antibody or preinfected with a DN-p38MAPK-β adenovirus (*DN-p38*) prior to treatment with or without TGF-β (200 pM for 16 h). The cells were then fixed and stained for ZO-1 and E-cadherin. Corresponding phase contrast images are shown with F-actin staining as *insets*. The p38MAPK expression and phosphorylation in TGF-β-treated NMuMG cells control (−) or DN-p38-infected (+) was determined by immunoblotting using β-tubulin as a loading control.

DNIIR expression was able to block TGF-β-mediated growth inhibition, SMAD-mediated transcriptional activation, and EMT. TGF-β-induced Smad2, and AKT phosphorylation was blocked by the expression of DNIIR. Yet, the induced expression of the cytoplasmically truncated TβRII at the levels that blocked the other measured responses did not block TGF-β-mediated p38MAPK activation. The potential for a lower TβRII threshold for p38MAPK activation was indicated by the results using cells with a TβRII null background, T47D. Blocking integrin-β_1-mediated adhesion resulted in the inhibition of TGF-β-mediated activation of p38MAPK. Further, TGF-β-mediated EMT was blocked both by the neutralizing integrin β_1 antibody and by limiting p38MAPK activity with a DNp38MAPK-β-expressing adenovirus. However, as DNIIR expressing cells are insensitive to TGF-β-mediated EMT progression, our data suggest that p38MAPK activation is necessary but not sufficient for the fibroblastic transition of NMuMG cells.

We investigated TGF-β signaling in NMuMG cells expressing an inducible *DNIIR* gene to assess the effect of a functional reduction of TβRII expression. The NMuMG cells were used as a model system for the expression of DNIIR because TGF-β induces both growth arrest and EMT in these cells (6, 28, 29). The expression of DNIIR at higher levels limited TGF-β-induced growth inhibition and diminished transcriptional induction of the luciferase reporters in NMuMG cells (Fig. 1). Similar DNIIR expression levels were also observed to block TGF-β-mediated Smad2/3 nuclear translocation, loss of ZO-1 tight junction expression, and the conversion to a fibroblastic morphology (Fig. 2). To identify signaling pathways that may be regulated in the presence of DNIIR expression subsequent studies were performed in the presence of 5 μM ponasterone. The results in Fig. 3 suggested that expression levels of DNIIR sufficient to desensitize the NMuMG cells to TGF-β-induced Smad2 and AKT activation still permitted TGF-β stimulation of p38MAPK phosphorylation. This result was consistent with the results of Chin *et al.* that showed TGF-β-mediated p38MAPK phosphorylation in cells expressing another cytoplasmically truncated construct of TβRII (30). Their expression of the mutant TβRII construct did not inhibit TGF-β induction of p38MAPK-mediated pro-α (1) collagen I mRNA expression in mesangial cells (30). However, since DNIIR expression was not able to rescue TGF-β-mediated p38MAPK phosphorylation in T47D cells, we speculate that a fraction of the receptor tetramer-ligand complex on the cell surface of NMuMG cells may include a combination of DNIIR as well as endogenous type I and type II receptors. Thus if sufficient over-expression of DNIIR was achieved in NMuMG cells one could anticipate the complete competition of TβRII and the loss of p38MAPK signaling.

We demonstrated that p38MAPK activation by TGF-β is dependent on integrin β_1 adhesion to ECM by examining TGF-β signaling in the presence of integrin β_1-neutralizing antibody. We also found that, integrin β_1-dependent adhesion was necessary for TGF-β-mediated p38MAPK activation in both DNIIR-expressing and -non-expressing NMuMG cells. Furthermore, our *in vitro* kinase results (Fig. 6) lend supportive evidence to the apparent cooperative regulation of p38MAPK by TGF-β and integrin β_1. TGF-β signaling to p38MAPK is thought to be mediated through the coupling of TAK1 and TAB for the subsequent recruitment of MKK3 and MKK6 and activation of p38MAPK (31, 32). Analogously the transient integrin-mediated activation of p38MAPK upon cell attachment on collagen I is mediated by Rac1-stimulated PAK association with MKK3 and MKK6 (19, 25). A potential mechanism for the cooperation of integrins and activated TGF-β receptor complexes may be their co-localization in caveolar vesicles (33, 34, 35). Another possibility is that integrin-dependent adhesion to ECM regulates scaffolding proteins that bring the signaling intermediaries together.

The microscopy results in Fig. 8 illustrate that blocking either integrin β_1 stimulation or inhibiting p38MAPK kinase activity can abrogate TGF-β-mediated EMT. Blocking integrin β_1 caused a phenotypic change independent of TGF-β that is possibly unrelated to p38MAPK activity. This was evident since the integrin β-neutralizing antibody alone resulted in the loss of ZO-1 junctional localization. There is recent evidence showing integrin β_1 co-localization together with apical tight junction-associated ZO-1 and occludin proteins (36). The disruption of this association may explain our result. Alternatively, the neutralizing antibody may cause a loss of cell polarity. E-cadherin, an epithelial marker localized at adherence junctions, was not affected by

blocking integrin-β_1 adhesion. Although the role of integrins in the maintenance of cell-cell junctions is not entirely clear and further complicates the interpretation of these results, it is evident that p38MAPK activity and integrin adhesion are necessary for TGF-β-induced EMT.

In conclusion we have presented data indicating that TGF-β can stimulate p38MAPK activation at a lower functional TβRII threshold than the activation of Smad2 and AKT. Furthermore, TGF-β-dependent p38MAPK activation is dependent on integrin β_1-ECM interactions and TGF-β-induced epithelial cell EMT is both p38MAPK activation- and integrin-β_1-mediated. Taken together these data suggest that functional TβRII dosage as well as cooperative signaling between the TGF-β receptor and integrins may influence the phenotypic fate of epithelial cells *in vivo*.

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REFERENCES

1. Brown, L. A., Yang, S. H., Hair, A., Galanis, A., and Sharrocks, A. D. (1999) *Oncogene* **18,** 7985–7993
2. Moses, H. L., Branum, E. L., Proper, J. A., and Robinson, R. A. (1981) *Cancer Res.* **41,** 2842–2848
3. Ebner, R., Chen, R. H., Shum, L., Lawler, S., Zioncheck, T. F., Lee, A., Lopez, A. R., and Derynck, R. (1993) *Science* **260,** 1344–1348
4. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) *Nature* **370,** 341–347
5. Zhang, Y., Feng, X., We, R., and Derynck, R. (1996) *Nature* **383,** 168–172
6. Bhowmick, N. A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C. A., Engel, M. E., Arteaga, C. L., and Moses, H. L. (2001) *Mol. Biol. Cell* **12,** 27–36
7. Atfi, A., Djelloul, S., Chastre, E., Davis, R., and Gespach, C. (1997) *J. Biol. Chem.* **272,** 1429–1432
8. Engel, M. E., McDonnell, M. A., Law, B. K., and Moses, H. L. (1999) *J. Biol. Chem.* **274,** 37413–37420
9. Wang, W., Zhou, G., Hu, M. C., Yao, Z., and Tan, T. H. (1997) *J. Biol. Chem.* **272,** 22771–22775
10. Griswold-Prenner, I., Kamibayashi, C., Maruoka, E. M., Mumby, M. C., and Derynck, R. (1998) *Mol. Cell. Biol.* **18,** 6595–6604
11. Krymskaya, V. P., Hoffman, R., Eszterhas, A., Ciocca, V., and Panettieri, R. A., Jr. (1997) *Am. J. Physiol.* **273,** L1220–L1227
12. Massague, J. (2000) *Nat. Rev. Mol. Cell. Biol.* **1,** 169–178
13. Ashcroft, G. S., Yang, X., Glick, A. B., Weinstein, M., Letterio, J. L., Mizel, D. E., Anzano, M., Greenwell-Wild, T., Wahl, S. M., Deng, C., and Roberts, A. B. (1999) *Nature Cell Biol.* **1,** 260–266
14. Bakin, A. V., Tomlinson, A. K., Bhowmick, N. A., Moses, H. L., and Arteaga, C. L. (2000) *J. Biol. Chem.* **275,** 36803–36810
15. Boudreau, N. J., and Jones, P. L. (1999) *Biochem. J.* **339,** 481–488
16. Kumar, N. M., Sigurdson, S. L., Sheppard, D., and Lwebuga-Mukasa, J. S. (1995) *Exp. Cell Res.* **221,** 385–394
17. Roberts, C. J., Birkenmeier, T. M., McQuillan, J. J., Akiyama, S. K., Yamada, S. S., Chen, W. T., Yamada, K. M., and McDonald, J. A. (1988) *J. Biol. Chem.* **263,** 4586–4592
18. Ignotz, R. A., and Massague, J. (1987) *Cell* **51,** 189–197
19. Mainiero, F., Soriani, A., Strippoli, R., Jacobelli, J., Gismondi, A., Piccoli, M., Frati, L., and Santoni, A. (2000) *Immunity* **12,** 7–16
20. Chen, R. H., Ebner, R., and Derynck, R. (1993) *Science* **260,** 1335–1338
21. Wang, Y., Huang, S., Sah, V. P., Ross, J., Jr., Brown, J. H., Han, J., and Chien, K. R. (1998) *J. Biol. Chem.* **273,** 2161–2168
22. Shipley, G. D., Tucker, R. F., and Moses, H. L. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4147–4151
23. Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F., and Massague, J. (1992) *Cell* **71,** 1003–1014
24. Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) *EMBO J.* **17,** 3091–3100
25. Klekotka, P. A., Santoro, S. A., and Zutter, M. M. (2001) *J. Biol. Chem.* **276,** 9503–9511
26. Guan, J. L., Trevithick, J. E., and Hynes, R. O. (1991) *Cell Regul.* **2,** 951–964
27. Weaver, V. M., Petersen, O. W., Wang, F., Larabell, C. A., Briand, P., Damsky, C., and Bissell, M. J. (1997) *J. Cell Biol.* **137,** 231–245
28. Miettinen, P. J., Ebner, R., Lopez, A. R., and Derynck, R. (1994) *J. Cell Biol.* **127,** 2021–2036
29. Piek, E., Moustakas, A., Kurisaki, A., Heldin, C., and ten Dijke, P. (1999) *J. Cell Sci.* **112,** 4557–4568
30. Chin, B. Y., Mohsenin, A., Li, S. X., Choi, A. M., and Choi, M. E. (2001) *Am. J. Physiol. Renal Physiol.* **280,** F495–504
31. Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E., and Hagiwara, M. (1996) *J. Biol. Chem.* **271,** 13675–13679
32. Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995) *Science* **270,** 2008–2011
33. Wary, K. K., Mariotti, A., Zurzolo, C., and Giancotti, F. G. (1998) *Cell* **94,** 625–634
34. Thorne, R. F., Marshall, J. F., Shafren, D. R., Gibson, P. G., Hart, I. R., and Burns, G. F. (2000) *J. Biol. Chem.* **275,** 35264–35275
35. Razani, B., Zhang, X. L., Bitzer, M., von Gersdorff, G., Bottinger, E. P., and Lisanti, M. P. (2001) *J. Biol. Chem.* **276,** 6727–6738
36. Tafazoli, F., Holmstrom, A., Forsberg, A., and Magnusson, K. E. (2000) *Infect. Immun.* **68,** 5335–5343